Title Page:
Constitutive Effects of Lead on Aryl Hydrocarbon Receptor Gene Battery and Protection by β-carotene and Ascorbic Acid in Human HepG2 Cells
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**ABSTRACT:**

Lead (Pb) is an environmental pollutant that can get entry into human body through contaminated foods, drinks and inhaled air leading to severe biological consequences, and has been responsible for many deaths worldwide. The objectives of this study were firstly to investigate the modulatory effects of environmentally relevant concentrations of Pb on AhR gene battery, which is controlling xenobiotics metabolism. Secondly, trials to reduce Pb-induced adverse effects were done using some phytochemicals like β-carotene or ascorbic acid. Human hepatoma (HepG2) cell lines were exposed to a wide range of Pb concentrations varying from physiological to toxic levels (0-10 mg/L) for 24 h. High Pb concentrations (1-10 mg/L) significantly reduced phase I (CYP1A1, 1A2) and phase II (UGT1A6 and NQO1) xenobiotic metabolizing enzyme mRNA expression in a mechanistic manner through the AhR regulation pathway. Additionally, These Pb concentrations induced oxidative stress in HepG2 cells in terms of production of reactive oxygen species (ROS) and induced heme oxygenase-1 mRNA expression in a concentration dependent phenomenon. Co-exposure of HepG2 cells to physiological concentrations of some micronutrients, like β-carotene (10 µM) or ascorbic acid (0.1 mM), along with Pb (1 mg/L) for 24 h significantly reduced the levels of ROS production and recovered AhR mRNA expression into the normal levels. Thus, consumption of foods rich in these micronutrients may help to reduce the adverse effects of lead in areas with high levels of pollution.

**Keywords:** AhR, ascorbic acid, β-carotene, lead, xenobiotic metabolism

**Practical Application:**
Lead modulated AhR gene battery in mechanistic way through AhR dependent pathway in human HepG2 cells. β-carotene and ascorbic acid can reduce the harmful effects of lead. Supplementation of carotenoids and ascorbic acid to people at highly polluted areas with lead can reduce the lead-adverse effects.
Introduction

Lead (Pb) is an environmental contaminant, which is detected at variable concentrations in different human food sources, including those of animal and plant origin. Pb is widely used in mining activities, electrical equipment, and manufacturing industries. Pb exists in the environment in a vapor or solid form, and is a ubiquitous, highly toxic, non-essential element. Studies from our laboratory and those of other groups recorded higher levels of Pb than the established maximum permissible limits in different human food items of animal and plant origin, including meat, edible offal, fish, poultry, and crops (Burger and others 2007; Yabe and others 2011; Ikenaka and others 2012; Morshdy and others 2013). Heavy metals, particularly Pb, are characterized by their persistent occurrence and accumulation (Korashy and El-Kadi 2004; ATSDR 2011). Humans are potentially exposed to these toxic metals from numerous sources such as food and water leading to several biological implications including xenobiotic metabolizing enzyme (XME) system leading to disruption of xenobiotic metabolic pathways (Hsu and Guo 2002; Masso-Gonzalez and Antonio-Garcia 2009).

Aryl hydrocarbon receptor (AhR)-regulated gene battery, mainly contribute to metabolism and detoxification of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs). This battery includes phase I enzymes such as cytochrome P450 (CYP) 1A1 and 1A2; phase II enzymes such as uridine diphosphate glucuronosyltransferase (UGT) 1A6 and NAD(P):quinone oxidoreductase 1 (NQO1) (Nebert and others 2000).

Co-contamination with mixtures of PAHs and heavy metals, such as Pb, is a worldwide environmental problem. These contaminants are co-released from different sources, such as
car exhausts, tobacco smoke, and fossil fuel combustion (McLemore and others 1990).

Disruption of the AhR gene battery by Pb can affect the mutagenicity and carcinogenicity of PAHs, HCAs, and TCDD (Vakharia and others 2001).

A number of recent studies have investigated the crosstalk between heavy metals and AhR. For example, Kluxen and others (2013) reported that cadmium induced the AhR-regulated gene battery in the rat small intestine and uterus. In addition, cadmium was shown to activate AhR, and AhR was required for GalNAc-T3 mRNA expression in osteoblast-like cells and subsequently induced fibroblast growth factor 23 (Kido and others 2014). Arsenite induced AhR-regulated enzymes in the kidney, lung, and heart of C57BL/6 mice (Anwar-Mohamed and others 2012). Mercury had a significant influence on the constitutive and TCDD-induced AhR-regulated genes in C57BL/6 mice in a time-, tissue-, and, AhR-regulated enzyme gene-dependent manner (Amara and others 2012). Copper modulated AhR and AhR-regulated gene mRNA expression in cultured rat H4IIE cells (Darwish and others 2014). Pb significantly downregulated TCDD-induced CYP1A1 mRNA, protein, and catalytic activity levels in HepG2 cells (Korashy and El-Kadi 2012). Moreover, in our previous report, a clear downregulation of AhR-regulated gene battery was recorded after Pb exposure of rat H4IIE cells (Darwish and others 2013). As a follow-up to our previous study, we planned this work to investigate the complex relationship between Pb and AhR gene battery in human liver HepG2 cells, especially under environmentally relevant concentrations of Pb. In addition, the mechanisms behind these effects were also examined.

Micronutrients such as β-carotene and ascorbic acid (As. Acid) are found naturally in vegetables, fruits, and green leafy plants. The antioxidant effects of these phytochemicals have
been reported previously in rodents, and they are frequently used as natural antioxidant substances in these animals. The antioxidant effects of these substances occur through reaction with free radicals, such as peroxyl, hydroxyl, and superoxide radicals, thus reducing or preventing oxidative damage to DNA, lipids, and proteins (Agarwal and others 2012; Chapman 2012).

The crosstalk between Pb, these micronutrients, and the AhR gene battery has not been investigated before. The present study was conducted to investigate the constitutive effects of Pb on phase I and II XME gene expression in human liver HepG2 cells. To explain the mechanisms underlying these effects, we examined the modulation of AhR mRNA and protein expression and luciferase activity after Pb exposure. Finally, the protective effects of micronutrients, such as β-carotene and As. Acid, against Pb-induced adverse effects were also examined.
**Materials and Methods**

**Chemicals and reagents**

All of the test reagents used were of reagent grade. TRI reagent, Dulbecco’s Modified Eagle’s Medium (DMEM), β-carotene, and goat anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Oligo(dT) primer, ReverTra Ace, and reverse transcriptase (RT) buffer were purchased from Toyobo (Osaka, Japan). Polyclonal rabbit anti-human AhR antibody was purchased from Abcam (Tokyo, Japan). Primer sets were purchased from Invitrogen (Carlsbad, CA). Lead acetate, As. Acid, and all other reagents were of analytical grade and purchased from Wako Pure Chemical Industries (Tokyo, Japan).

**Cell line and culture conditions**

All experiments were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. The human hepatoma cell line HepG2, obtained from RIKEN Cell Bank (Tsukuba, Japan), was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in a humidified incubator with 5% CO2. Cells were seeded in 60-mm collagen-coated dishes, subcultured twice a week, and subsequently grown to 80% – 90% confluence. Cells were exposed to lead acetate (Pb) (0 – 10 mg/L) in serum-free medium for 24 hours. In cell viability protection experiments, HepG2 cells were exposed to Pb (1 mg/L) or to β-carotene (1 or 10 µM) alone or 3 hours after Pb exposure. HepG2 cells were also exposed to As. Acid (0.1 or 1 mM) alone or 3 hours after treatment with Pb. In antioxidant protection experiments, cells were exposed to either β-carotene (10 µM) or As. Acid (0.1 mM) alone or 3 hours after...
treatment with Pb. After cell treatments for 24 hours, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS).

**Cell viability assay**

Cell viability was examined using the CCK-8 assay (Sigma-Aldrich, St. Louis, MO) according to manufacturer’s instructions.

**RNA extraction & cDNA synthesis**

Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. Total RNA concentration and quality were checked using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, CT). The RNA quality was estimated by the 260/280 nm and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis.

The cDNA was synthesized as described previously (Darwish and others 2010). Briefly, a mixture of 5 µg of total RNA and 0.5 ng of oligo(dT) primer in a total volume of 7 µL of sterilized ultra-pure water was incubated at 70°C for 10 minutes and then removed from the thermal cycler. The volume was increased to 20 µL with a mixture of 4 µL (5x) RT-buffer (Toyobo Co., Ltd, Osaka, Japan), 8 µL of 10 mM dNTPs, and 1 µL of reverse transcriptase (Toyobo). The mixture was then reincubated in the thermal cycler at 42°C for 45 minutes and at 90°C for 10 minutes to prepare the cDNA.

**Quantitative real-time polymerase chain reaction**

Quantitative real-time PCR for human mRNA levels was performed using StepOne™ Real-Time PCR System (Applied Biosystems). The primer sequences are described in Table 1 (Ohno and Nakajin 2009; Amara and others 2010). PCR was performed in a volume of 10 µL
according to the protocol described previously (Mureithi and others 2012). Briefly, the PCR mixture was prepared with SYBR® qPCR Mix (Toyobo), 10 µM of each primer, 600 ng of cDNA, and 50× ROX reference dye, and then made up to a final volume of 10 µL with RNase-free water. The reaction cycle started with an initial holding stage at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 minute, and extension at 72°C for 30 s. Confirmation of a single amplicon amplification at the expected size was confirmed through melting curve analysis and agarose gel electrophoresis. β-actin was used for normalization in the comparative Ct method.

Detection of protein expression and Western blotting

Total protein extracts were prepared according to the method described before (Ceckova and others 2006). Protein concentrations were measured using a BCA assay kit (Pierce Biotechnology, Rockford, IL). Aliquots of 12 µg of protein were used for detection of protein expression in each treatment. Following SDS-PAGE separation and Western blotting, blots were probed with primary antibody against AhR (polyclonal rabbit anti-human antibody). Chemiluminescence detection was performed using secondary antibody (goat anti-rabbit antibody) conjugated with horseradish peroxidase and an Amersham (GE Healthcare, Waukesha, WI) ECL kit. The intensity of bands was quantified using the public domain NIH Image program (National Institutes of Health, Bethesda, MD).

Luciferase Assay

A luciferase assay was performed using H4IIE-XRE cells according to the method described previously (Ohno and others 2012a). Briefly, one day before the experiment, cells were seeded in 96-well plates in DMEM supplemented with 10% FBS. The growth medium was
removed and replaced with Pb (0 – 10 mg/L) or Sudan III (10 μM) as positive control alone or combined with Pb at 10 mg/L. Twelve hours after exposure, the medium was aspirated off and luciferase assay was performed using the Dual Glo luciferase assay system (Promega, Madison, WI) according to the manufacturer’s protocol. After incubation of the substrate with cell lysate, the luminescence was measured at 590 nm using a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany).

**Determination of reactive oxygen species (ROS)**

The fluorogenic probe 2’,7’-dichlorofluorescein diacetate (DCF-DA) (Sigma) was used for ROS measurement as described previously (Korashy and El-Kadi 2012). Briefly, HepG2 cells were incubated with the test compounds for 24 hours. The cells were then incubated with DCF-DA (5 μM) for 1 hour at 37°C. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a 96-well plate reader (Baxter, Deerfield, IL).

**Statistical analysis**

Statistical significance was evaluated using the Tukey–Kramer HSD test (JMP statistical package; SAS Institute Inc., Cary, NC). In all analyses, P < 0.05 was taken to indicate statistical significance.
Results and Discussion

Exposure of humans to Pb is very common worldwide either via ingestion of contaminated foods, drinks or inhalation in Pb polluted areas. Recently, Pb is encountered in many poisoning cases especially in children in many locations such as in Bagega community, Zamfara, Nigeria (Ajumobi and others 2014); in Kabwe, Zambia (Yabe and others 2014) and in Changchun, Jilin Province, China (Xu and others 2014). The first objective of this study was to investigate the constitutive effects of Pb (under relevant concentrations found in environment) on XMEs in the human HepG2 cells, especially, AhR gene battery. Pb (0-5 mg/L) did not alter cell viability, however, only Pb (10 mg/L) significantly reduced cell viability with 29% (Fig. 1).

The obtained results in figure 2 showed that, Pb under low concentration (0.5 mg/L) could induce CYP1A1 and NQO1 mRNA expression levels (Fig. 2A, D). Relatively higher concentrations (1-10 mg/L) of Pb, clearly down regulated CYP1A2 and UGT1A6 mRNA expressions (Fig 2B, C); human CYP1A1 and NQO1 expressions were down regulated under only 10 mg/L exposure. These results were corresponding to the down regulation of XMEs of rat H4IIE cells when exposed to elevated concentrations of Pb (Darwish and others 2013). Additionally, Korashy and El-Kadi (2012) recorded a strong reduction in the induction level of human CYP1A1 mRNA expression when human liver cells were co-exposed to TCDD (potent inducer for CYP1A1) and Pb (100 µM). AhR gene battery is regulated through AhR receptor, which is usually found in inactive form in the cytoplasm. AhR is activated upon binding with AhR-ligands such as TCDD or benzo[a]pyrene (BP) forming a ligand receptor complex which translocates into the nucleus and dimerizes with Aryl hydrocarbon nuclear translocator (ARNT). This complex by turn binds with xenobiotic responsive element (XRE) located in the promoter region of each AhR
regulated genes resulting in release of what is called AhR gene battery (Whitlock 1999).

However, the modulation of AhR gene battery as declared in figure 2 is occurred in the absence of any AhR ligand. In order to explain that, we investigated the effects of Pb on the AhR mRNA, protein and luciferase activity as in figure 3 (A, B and C). The obtained results showed that Pb slightly induced AhR mRNA and protein expressions under the low Pb concentrations (0.25 and 0.5 mg/L) (Fig. 3A, B). Interestingly, AhR-XRE luciferase activity was also induced under 0.5 mg/L Pb exposure (Fig. 3C). This interesting finding suggests a crosstalk between Pb and AhR, despite of its different structure than AhR-typical planar, aromatic ligands as BP and dioxins.

Induction of AhR and AhR-gene battery with non-typical AhR ligands was recorded previously with sudan III, flavonoids, endogenous tryptophan derivatives, or by the antioxidant tert-butylhydroquinone (tBHQ) (Schreiber and others 2006; Refat and others 2008; Ohno and others 2012b; Vaas and others 2014). However, higher concentrations of Pb (1-10mg/L) significantly reduced AhR mRNA and protein expressions. That was clear when Pb reduced the strong luciferase activity of sudan III (potent AhR ligand, Ohno and others 2012b) (Fig. 3C).

These results were corresponding to the effects of Pb on XMEs expression (Fig. 2). Thus, we can suggest that the modulatory effects of Pb on XMEs is possibly due to Pb-AhR crosstalk.

In order to explain the strong cytotoxic and genotoxic effects of Pb on AhR and AhR gene battery especially, under higher concentrations, we examined the oxidative stress induction by Pb. Lead induced ROS production in HepG2 cells in concentration dependent fashion (Fig. 4A). In parallel, Pb also induced HO-1 mRNA expression (Oxidative stress marker) (Fig. 4B). Thus, induction of oxidative stress may explain the cytotoxic and genotoxic effects of Pb especially under the higher concentrations (1-10 mg/L). This finding goes in agreement with
Korashy and El-Kadi (2012), who recorded a clear induction of HO-1 mRNA and ROS production in HepG2 cells co-exposed to TCDD and Pb.

In a trial to reduce these cytotoxic and genotoxic effects of the Pb on XMEs, HepG2 cells were co-exposed to some micronutrients such as β-carotene and As. Acid with different concentrations. Figure 5 showed a significant concentration dependent improvement in the cell viability after addition of either β-carotene or As. Acid to cells treated with Pb 10 mg/L. In addition, the results obtained in figure 6 (A & B), showed a clear reduction of Pb-induced ROS after adding either β-carotene (10 µM) or As. Acid (0.1 mM) to cells exposed to Pb 1 mg/L. Reduction of ROS by β-carotene is going in agreement with Kasperczyk and others (2014), who observed reduction of ROS in lead exposed-workers after β-carotene supplementation in their diets.

Additionally, we investigated the effects of co-exposure of Pb (1 mg/L) and β-carotene or As. Acid on AhR mRNA. Interestingly, we observed that β-carotene, which is a mild AhR ligand (Darwish and others 2010), significantly recovered AhR mRNA expression, that may be due to competition for the substrate binding sites or due to the radical scavenging effects (Fig. 7A). Similarly, As. Acid, which is non-AhR ligand, strongly and significantly recovered AhR mRNA expression to the normal level (Fig. 7B), that could be due to the strong radical scavenging effects of As. Acid. Thus, we highly encourage people living in Pb polluted areas to consume foods rich in β-carotene and As. Acid, which will be helpful to counteract the genotoxic effects produced by Pb.
Conclusion

This study indicated that environmentally relevant concentrations of Pb could modulate XME expression profiles in a mechanistic dependent manner. Interference of Pb with AhR metabolic pathways may represent a public health concern, especially in areas highly polluted with dioxins, PAHs, and HCAs, leading to increased mutagenicity and carcinogenicity of such substances. Dietary supplementation with some micronutrients like β-carotene and As. Acid has significant beneficial roles in reducing the risks of human exposure to Pb.

Acknowledgments

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Author Contributions

W. S. Darwish conducted the experiments, drafted the manuscript and interpreted the results. Y. Ikenaka designed the study and interpreted the results. S. M. Nakayama drafted the manuscript, interpreted the results and performed statistical analysis. M. Ishizuka designed the study, supervised the work and interpreted the results.
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Table 1: Primer sequences of the target genes used in this study

<table>
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<tr>
<th>Target</th>
<th>Sequence</th>
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<tr>
<td>Human CYP1A1</td>
<td>F: 5'-CTATCTGGGCTGTGGGCAA-3'</td>
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<td></td>
<td>R: 5'-CTGGCTCAAGCACAACCTTG-3'</td>
</tr>
<tr>
<td>Human CYP1A2</td>
<td>F: 5'-CATCCC CCACAGCACAGCAAGCAAG-3'</td>
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<td>R: 5'-TCCCACTTGCCAGGACTTC-3'</td>
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<td>Human UGT1A6</td>
<td>F: 5'-CATGATTGTATTGGGCTGTAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TCTGTGAAAAGAGCATCAAAACT-3'</td>
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<tr>
<td>Human NQO1</td>
<td>F: 5'-GGATTGGCCCGAGCTGGAA-3'</td>
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<tr>
<td></td>
<td>R: 5'-AATTGACATGAAGATGAAGGCAAC-3'</td>
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<tr>
<td>Human HO-1</td>
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<td>R: 5'-TGTTGCGCTCAATCTCCTCCT-3'</td>
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<td>Human AhR</td>
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<tr>
<td>Human β-actin</td>
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Figure 1: Effects of Pb on HepG2 cell viability
Figure 2: Phase I and II mRNA expression in human HepG2 cells exposed to Pb

The effects of Pb treatment on: A) CYP1A1, B) CYP1A2, C) UGT1A6, and D) NQO1 mRNA expression in HepG2 cells using real-time qPCR. The cDNA samples were amplified as described in the Materials and Methods section. The amount of each enzyme was normalized relative to the corresponding amount of β-actin and presented relative to cells treated with water. Each treatment was replicated in five plates (n=5). Data are presented as the means ± standard deviation (SD). Identical letters indicate no significance difference from each other (P < 0.05).
Figure 3: Effects of Pb on AhR mRNA, protein expression and luciferase activity in HepG2 cells

The effects of Pb treatment on: A) AhR mRNA expression in HepG2 cells. The cDNA samples were amplified as described in the Materials and Methods section. The amount of AhR mRNA was normalized relative to the corresponding amount of β-actin and presented relative to cells treated with water. Each treatment was replicated in five plates (n=5). B) AhR protein expression was detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and an ECL kit. Each treatment was replicated in five plates (n=5). C) AhR-XRE luciferase activity in HepG2 cells. Luciferase assay was performed using H4IIE-XRE. Cells were treated with Pb (0 – 10 mg/L), or with Sudan III (10 μM) as a positive control alone or combination with 10 mg/L Pb. Twelve hours after exposure, the medium was aspirated off and luciferase assay was performed using the Dual Glo luciferase assay system. After incubation of the substrate with cell lysate, the luminescence was measured at 590 nm using a Mithras LB940. Each treatment was replicated in five wells (n=5). All experiments were repeated at least three times on different days. Data are presented as the means ± SD. Identical letters indicate no significance difference from each other (P < 0.05).
Figure 4: Induction of oxidative stress in HepG2 cells after Pb exposure

A) ROS production in HepG2 cells exposed to Pb (0 – 10 mg/L). Cells were incubated with DCF-DA (5 µM) for 1 hour at 37°C. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Baxter 96-well plate reader. Each treatment was replicated in five wells (n=5). B) Heme oxygenase-1 (HO-1) mRNA expression in HepG2 cells. The cDNA samples were amplified as described in the Materials and Methods section. The amount of HO-1 mRNA was normalized relative to the corresponding amount of β-actin and presented relative to cells treated with water. Each treatment was replicated in five plates (n=5). Data are presented as the means ± SD. Identical letters indicate no significance difference from each other (P < 0.05).
Figure 5: Protective effects of β-carotene and ascorbic acid against lead-induced cytotoxicity

The effects of adding either β-carotene (1 or 10 µM) or ascorbic acid (0.1 or 1mM) to HepG2 cells 3 hours after treatment with Pb at 10 mg/L. Cell viability was examined using a CCK-8 assay kit as described in the Materials and Methods section. Data are presented as the means ± SD. Identical letters indicate no significance difference from each other (P < 0.05).
Figure 6: Protective effects of β-carotene and ascorbic acid against lead-induced oxidative stress

A) HepG2 cells were exposed to Pb 1 mg/L or β-carotene (10 µM) alone or 3 hours after exposure to Pb. B) HepG2 cells were also exposed to ascorbic acid (0.1 mM) alone or 3 hours after treatment with Pb. After treatment of cells for 24 hours, the medium was removed, and the cells were washed twice with PBS. Cells were incubated with DCF-DA (5 µM) for 1 hour at 37°C. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a Baxter 96-well plate reader. Each treatment was replicated in five wells (n=5). Data are presented as the means ± SD. Identical letters indicate no significance difference from each other (P < 0.05).
Figure 7: Recovery effects of β-carotene and ascorbic acid against lead-induced genotoxic effects on AhR mRNA expression

A) HepG2 cells were exposed to Pb 1mg/L or β-carotene (10 µM) alone or 3 hours after exposure to Pb. B) HepG2 cells were also exposed to Pb 1 mg/L or ascorbic acid (0.1 mM) alone or 3 hours after treatment with Pb. After treatment of cells for 24 hours, the medium was removed, and the cells were washed twice with PBS. RNA was extracted, and cDNA was synthesized. AhR mRNA expression was estimated by real-time PCR as described in the Materials and Methods section. Each treatment was replicated in five wells (n=5). Data are presented as the means ± SD. Identical letters indicate no significance difference from each other (P < 0.05).